

BEHAVIORAL FREEZE AVOIDANCE IN AN ANTARCTIC FISH

BY

MATEUSZ GROBELNY

THESIS

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Master's Committee:

Professor Christina Cheng, Director of Research  
Professor Arthur DeVries  
Assistant Professor Julian Catchen

## ABSTRACT

Freeze avoidance is vital for the survival of Antarctic notothenioid fishes inhabiting the ice-laden waters of the high-latitude Southern Ocean. Most notothenioids avoid freezing primarily by the action of their blood-borne antifreeze glycoproteins (AFGPs). AFGPs arrest the growth of environmental ice crystals that enter the fish, thereby preserving the body fluids in liquid state. AFGPs are ubiquitous in all adult Antarctic notothenioids examined, except for an early report that one species, *Lepidonotothen squamifrons* (from Balleny Islands waters near the Ross Sea) lacked measurable AFGP activity or thermal hysteresis (TH). How it survives in the ice-covered Southern Ocean is unclear. Analyses of the sera of *L. squamifrons* from the West Antarctic Peninsula (WAP) confirmed that TH was indeed minimal or absent. The sera osmolality was  $429 \pm 19$  mOsm/Kg equivalent to a freezing point depression (f.d.p) of  $0.8^{\circ}\text{C}$  while the hysteresis (melting point-freezing point) was only  $0.017^{\circ}\text{C}$ . The combined f.p.d of  $0.817^{\circ}\text{C}$  was well above the freezing point of seawater ( $-1.9^{\circ}\text{C}$ ) which puts this species at risk of freezing should it encounter ice-laden freezing seawater. To determine whether *L. squamifrons* might be using an alternative freeze avoidance strategy, by inhabiting only the warm ice-free waters beneath the freezing surface layer, their thermal preference was tested by placing specimens with an attached temperature logger in a horizontal thermal gradient aquarium ( $-1^{\circ}\text{C}$  to  $+1^{\circ}\text{C}$ ), along with AFGP-fortified species used as a control. *L. squamifrons* exhibited the warmest preferred median temperature at  $0.78^{\circ}\text{C}$  while AFGP fortified species preferred lower water temperatures. Expendable bathythermograph (XBT) casts and historical water column profiles at *L. squamifrons* catch sites indicated that their habitat temperatures were well above freezing, suggesting this species preferred non-freezing water temperatures in the wild. This is the first demonstration of a behavioral freeze avoidance in an Antarctic notothenioid fish as a freeze avoidance strategy in the frigid Southern Ocean.

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## INTRODUCTION

Teleost fish of the high latitude Southern Ocean faced the risk of freezing when the ocean cooled and sea ice began to form. The onset of those extreme conditions, around 15 Mya (Zachos et al., 2001), lead to a mass extinction of most of the marine fauna in the region, however the ancestral perciform notothenioids were able to adapt (Eastman, 2004). The success of the notothenioids has been attributed to the evolution of adaptations that lead to rates of biochemical and physiological processes compatible with rates of growth and reproduction similar to those in cold-water temperate teleosts. One of the critical adaptations was the evolution of antifreeze glycoproteins (AFGPs) (DeVries, 1988; Verde et al., 2006). Today the Notothenioidie represent 77% of the species and 91% of the biomass of the Antarctic continental shelf ichthyofauna (Eastman, 2004) attesting to their success in adapting to this freezing environment.

The discovery of AFGPs in the late 1960s indicated that notothenioid survival in the freezing Antarctic waters was associated with high levels of AFGPs in the blood (~30-40mg/ml) (DeVries and Wohlschlag, 1969). AFGPs inhibit the growth of internalized ice crystals by an absorption-inhibition mechanism which effectively lowers the non-colligative freezing point (DeVries, 1988; Raymond and DeVries, 1972) . This unique property in conjunction with the colligative freezing point depression due to blood dissolved solutes, mainly NaCl, prevent the notothenioids from freezing (DeVries, 1988). Since freezing avoidance is critical for survival, AFGPs are found in almost all adult notothenioids (DeVries, 1988) having evolved prior to the radiation of the notothenioids across the Southern Ocean (Matschiner et al., 2011). The efficacy of AFGPs as a mechanism for freeze avoidance is highlighted by their occurrence in the unrelated gadid fishes (cods) inhabiting the freezing waters of the northern hemisphere (Chen et al., 1997; Cheng & DeVries 1989) which has been referred to as a classic example of convergent evolution at the protein level.

However, an early report suggested an interesting exception to the widespread occurrence of AFGPs in Antarctic fishes in that the notothenioid *Lepidonotothen squamifrons*, a grey rock cod, from Balleny Islands waters near the Ross Sea lacked a measurable AFGP activity (DeVries and Lin 1977). Furthermore, a recent study which surveyed the blood sera of a *L. squamifrons* population from sub-Antarctic islands confirmed the absence of any significant AFGP activity and a relatively low melting point compared to other notothenioids (Miya et al., 2016). Without protective AFGPs, *L. squamifrons* would be susceptible to freezing and incapable of surviving the

icy, freezing waters of the Southern Ocean. How this species avoids freezing in the freezing ocean is unexplained.

In their early report, DeVries & Lin (1977) proposed that the benthic *L. squamifrons*' low latitudinal distribution may avoid freezing by inhabiting a layer of warm water overlain by freezing ice-laden seawater. Koubbi *et al.* (2000) reported that the distribution of *L. squamifrons* is confined to the waters of the low latitude sub-Antarctic islands and waters adjacent the northern part of the WAP which would be consistent with the proposed freeze avoidance strategy. In addition, year round warm bottom water (0°C) is associated with approximately 13% of the continental shelf along the WAP (Clarke *et al.*, 2009) such that the benthic water temperatures along WAP consist of a bottom layer of relatively warm water overlain with surface freezing water during the winter.

To determine if *L. squamifrons* avoids freezing by residing in nonfreezing water by a behavioral freeze avoidance strategy we set out to assess its preferred temperature. The preferred temperature of an organism can be determined in the framework of the thermal preferendum paradigm which assumes that an organism will select a water temperature which is optimal for its physiological and biochemical processes. It is believed to be species-specific, and unaffected by short term acclimation (Reynolds and Casterlin, 1979). By placing *L. squamifrons* and control AFGP-fortified fishes in a thermal gradient aquarium with a +1°C to -1°C range it was possible to ascertain their thermal preference. Thus, we hypothesized that when placed in a thermal gradient aquarium *L. squamifrons*, with low or unmeasurable AFGP activity, will exhibit a thermal preference for water temperatures above the freezing point of its blood sera (-0.8°C) or water temperatures generally warmer than control AFGP-fortified fishes; *Notothenia coriiceps*, *Chaenodraco wilsoni* and *Trematomus hansonii* - which may have preference for subzero waters or demonstrate a larger range of preferred temperatures as they would not be in danger of freezing at the low temperature end of the gradient (Bilyk and DeVries, 2009; Jin, 2003; Jin and DeVries, 2006).

## METHODS

### *Collection of Fish*

Specimens of *L. squamifrons* were collected by trap in the Bismark Strait at the southern tip of Anvers Island WAP in 200m of water during the austral winters of 2013 and 2014. Twelve specimens were captured during 2013 and six during July in 2014 and were used to determine blood serum osmolality and AFGP activity. Specimens used for the temperature preference experiments were captured during the austral winter, during July-August 2014 (Table 1). *C. wilson*, *T. hansonii* and *L. squamifrons* were caught using a combination of benthic trawling (~300m) and baited traps deployed from the R/V *Laurence M. Gould*. They were held in running seawater aquaria (approximately -1.00°C), until they were transferred to Palmer Station aquaria (approximately -1.00°C), except *L. squamifrons* which was kept at approximately 0°C because of the risk of freezing at temperatures below their freezing point of ~-0.8°C. *N. coriiceps* were caught by rod fishing from the shore of Palmer Station where the water temperature was ~ -1.8°C and held in aquaria at -1°C. Prior to experimentation, each fish had a minimum week long acclimation period in the holding tanks.

### *Thermal Hysteresis*

To quantify the AFGP activity in *L. squamifrons*, blood samples were collected from the caudal vein with a 5ml syringe using a 23-gauge needle. The blood was allowed to clot at +4°C, then centrifuged and the blood serum stored at -80°C until analyzed at the University of Illinois. The melting and freezing points (MP and FP) were determined with a Clifton Nanoliter Osmometer (Clifton Technical Physics, Hartford, NY, USA), and the difference between the two points was used as a measure of antifreeze activity (thermal hysteresis) (Cziko et al., 2006). MP and FP points were determined according to Cziko *et al.*'s (2006) methodology. To measure the melting and FP, the wells on a microscopic cryo-stage were filled with heavy immersion oil using a glass micropipette syringe. Using a glass micropipette connected to a mineral oil-filled micrometer syringe, 2-5 nanoliters of serum was introduced into each of the seven wells containing immersion oil. The cryo-stage was cooled to -40°C and the frozen sample slowly warmed until only a single, 5-20  $\mu\text{m}$  diameter ice crystal remained. The MP was taken as the temperature at which the crystal melted. The FP value was the temperature at which a small crystal began to grow when cooled at a rate of  $0.074^\circ\text{C} \cdot \text{min}^{-1}$  after an annealing period of 60 seconds slightly below the MP. Three measurements were collected per sample. Wells were selected based on the order

of freezing during flash freezing (the first well surveyed was the first well to freeze during the flash freezing etc). The non-colligative freezing point depression was calculated from blood serum osmolality using:

$$\text{freezing point depression } ^\circ\text{C} = \left( \frac{x \text{ mOsm/Kg}}{1000} \right) - 1.86^\circ\text{C} \text{ (DeVries and Cheng, 2005)}$$

#### *Blood serum osmolality*

Blood serum osmolality for *L. squamifrons* was determined in triplicate using a Wescor 5520 osmometer calibrated with 290 and 1000 mOsm/Kg standards - the average of the two closest values for each standard and serum sample were averaged. Furthermore, *C. wilsoni*, *T. hansonii* and *N. coriiceps* TH and osmolality data was gathered from Bilyk and DeVries (2009), Grady and DeVries (1982), Jin (2003) and Jin and DeVries (2006) to draw comparisons between AFGP-fortified species and *L. squamifrons* (Appendix A).

#### *Thermal Gradient and Thermal Preference*

To determine the thermal preference of *L. squamifrons*, *C. wilsoni*, *T. hansonii* and *N. coriiceps*, a thermal gradient tank was set up in a cold room at Palmer Station chilled to  $-3.0^\circ\text{C}$ . The tank consisted of a wooden box 320cm long by 35cm and 31cm deep, lined with two layers of translucent plastic and contained ~240L of sea water. Silicon air tubing connected to a Supra 1 airpump (Second Nature) with small holes every 10 cm ran along the length of the tank to provide aeration. A VWR Scientific Products Immersion Circulator model 1122 set to  $+1.00^\circ\text{C}$  was placed at the warm end of the gradient. At the cold end a 6.1 m x 2.1 cm diam. copper coil was connected to a Neslab RTE Refrigerated circulator filled with ~20% ethylene glycol and set to  $-5.5^\circ\text{C}$ . A HOBO UX120 Thermocouple (Onset Computer Corp.) four-channel temperature logger with four thermocouple sensors was used to record the temperatures in the thermal gradient. The four thermocouple sensors (A, B, C, and D) were spaced at 107 cm intervals starting from the warm end of the aquarium. Together, the tank, heater and chiller resulted in a stable  $+1^\circ\text{C}$  to  $-1^\circ\text{C}$  thermal gradient (Fig. 1) although not a linear gradient (Supplemental Fig.1).

A temperature logger was attached to each specimen to track the temperature of their position in the temperature gradient with time. Each logger consisted of a HOBO UA-002-64 Pendant Temperature Logger (Onset Computer Corp.) which was prepared by initiating temperature logging using the HOBOWare Software (Onset Computer Corp.) with recording rate and units set to  $^\circ\text{C} \cdot \text{sec}^{-1}$ . A short piece of string was attached to the logger which was secured to the fish by



inserting a short clothing tag through the knotted end of the string into the dorsal muscle mass behind the first ray of the 2<sup>nd</sup> dorsal fin.

To determine the thermal preference specimens with attached temperature logger were randomly placed at either the warm or cold end of the gradient. Subsequently, the fish was left undisturbed for three<sup>1</sup> or more hours in the dark. Specimens were removed after 3 hours and the total length in cm and weight in grams recorded. In addition, the *L. squamifrons* specimens were bled and their blood serum frozen at -80°C. The thermal preference was determined for 6 specimens of each species with one individual tested at a time in the tank. Following six runs, the gradient tank water was replaced.

#### *Thermal Gradient Data Processing*

After each trial, the temperature data was downloaded from the logger using the HOBOWare Software, initially saved as a raw file and exported to a CSV format. The raw data values that were outside of those of the thermal gradient as well as the the first 500 and last 200 data points were trimmed by as they together represented temperatures recorded during set up and removal of the specimen logger. To determine the thermal preference of each species the analysis of the temperature time series data involved four consecutive steps; (1) transformation of the temperature series data into probabilities using the empirical distribution function<sup>2</sup> (EDF) of the four species, (2) comparison of the initial temperature range ( $T_0$ ) and final temperature range ( $T_{end}$ ) for each species, (3)  $T_{end}$  range comparison between species and (4)  $T_{end}$  range direction comparison between species. Significant differences between species EDFs would indicate a temperature preference as opposed to random movement in the gradient. If each temperature time series EDF is unique, then the second, third and fourth steps, would determine the temperature range each species preferred and how preferred temperatures differed between species.

#### *Empirical Distribution Functions*

To determine whether a species movement in the temperature gradient was random during the 3-hour exposure, the temperature time series data for the six individuals of each species were pooled and transformed into probabilities of occupying a position in the temperature gradient (cumulative probability EDF). To determine if the species EDF distributions were significantly different from each other the Kolmogorov-Smirnov (Hesamian and Chachi, 2015) non-parametric

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<sup>1</sup> Initially longer experimental durations were utilized however, after several preliminary trials most fish settled down to a single temperature within in three hours.

<sup>2</sup> Empirical distribution function is the fraction of y values less than or equal to the value x vs x.

test was applied which compares the greatest distance between the cumulative distributions between species in a pair wise test.

#### *Initial and Final Temperature Range Analysis*

In addition to the EDF analysis, the temperature ranges occupied during initial 500 sec ( $T_0$ ) and the final 500 sec ( $T_{end}$ ) were compared to determine whether a given species had moved to a different temperature than near the beginning of the temperature gradient exposure. Since each fish specimen was randomly placed at either end of the thermal gradient at the beginning of the experiment, then the median of temperature ranges occupied in the initial 500 sec and final could be compared and different medians of the  $T_{end}$  data range would be indicative of the species' preferred temperature. The median value was chosen as the main summary statistic as it is more robust in approximating the most common value in the data set while being less susceptible to the effect of extremely high and low values.

The  $T_0$  and  $T_{end}$  temperatures of the 6 specimens for each species were separately pooled with the assumption that intraspecies variation of temperature preference was consistent within a species. The distributions of the pooled data  $T_0$  vs  $T_{end}$  with the medians and interquartile was tested using the Wilcoxon rank sum non-parametric test, which tests for the null hypothesis that two different groups have a similar distribution of values without assuming a normal distribution of the input data.

#### *Final Temperature Range Comparison Between Species*

After the EDF and the comparisons of the initial and final temperature range, the  $T_{end}$  pooled data for each species was used to perform an interspecies comparison to determine if the preferred temperature range was different between species. The Kruskal-Wallis two-side non-parametric test was used to determine if the mean ranks for the  $T_{end}$  range for each species differed. The null distribution was approximated using Monte Carlo resampling of the input dataset with 10,000 replicates

#### *Comparison of Final Gradient Positions*

To determine each species' preferred temperature range in the gradient, Dunn's Kruskal-Wallis Multiple Comparisons post hoc test was used. Comparison were made between each species'  $T_{end}$  pooled data and the minimum, first quartile, median, third quartile, maximum and interquartile range values for each species  $T_{end}$  pooled data was recorded.

### *Data Processing– R packages*

Each step of the Data Processing analysis was performed in R with the Rstudio IDE. Steps one and two used the R Stats package while step three used the Coin package and step four used the FSA package for analysis. R scripts used to perform the analysis and generate graphs and will be available on: [GITHUB link]

### *Catch Site Water Column Thermal Profiles*

Water column thermal profiles were obtained using the Expendable bathythermographs (XBTs) (Lockheed Martin). XBT casts were done in the Bismark Strait (-64 52.45801, -63 39.5166), Dallman Bay (-63 55.85107 -62 48.50049), Gerlache Strait (-64 43.35596, -63 1.41016) following the manufacturers protocol. Each XBT was cast off the port side of the R/V *Laurence M. Gould* while underway.

### *Catch Specific Year-round Water Column Profiles*

To determine if there were nonfreezing water temperatures at the *L. squamifrons* catch sites throughout the year, water column temperature data for latitudes -59 0.0 to -76 0.0 and longitudes -82 30.0 to -49 30.0 was extracted from the following: Ocean Station Data, Conductivity-Temperature-Depth, Expendable Bathythermograph Data, Mechanical Bathythermograph Data, Profiling Floats Data, Autonomous Pinniped Bathythermograph Data and Glider Data was gathered from the World Ocean Database 2013 (NOAA). A water column thermal profile with temperatures averaged per standard increments of depth for each month was generated from data within a radius of 1.0 decimal degrees of the *L. squamifrons* catch locations (Dallman Bay, Hugo Island Basin, Bismark Strait and Gerlache Strait). This analysis was performed in Python using a custom script. [GITHUB link]

## RESULTS

### *Thermal hysteresis and osmolality*

*L. squamifrons*' blood serum osmolality was  $429 \pm 19$  mOsm/Kg, equivalent to a colligative freezing point depression of  $0.80 \pm 0.0036^{\circ}\text{C}$  ( $n = 18$ ). The melting point/freezing point difference (TH) determined with the Clifton Nanoliter Osmometer was  $0.017 \pm 0.0021^{\circ}\text{C}$  (Fig. 2 & Sup table 1). The approximate diameter of the seed ice crystal was  $15.9 \pm 0.96 \mu\text{m}$  (Fig. 3). During growth, very little faceting was observed.

### *Thermal Gradient Data - Temperature Time Series EDF Analysis*

To determine if each species' temperature time series data represented unique movement within the thermal gradient the EDFs for each species' were plotted to visualize the probabilities of residing at a given temperature (Fig. 4). *N. coriiceps* mostly resided in colder water as more than 75% of recorded temperatures were below zero. *T. hansonii* had a uniform distribution of probabilities across the temperature gradient suggesting that it may have moved randomly in the gradient. *C. wilsoni* exhibited a similar pattern to that of *T. hansonii* at the lower ranges  $< 0.5^{\circ}\text{C}$  but, at temperatures  $> 0.5^{\circ}\text{C}$  exhibited a spike in the probability of finding a temperature of  $0.5^{\circ}\text{C}$  or more. Lastly, *L. squamifrons* had the most precipitous drop in the probability of residing at temperatures below  $0^{\circ}\text{C}$  and the highest probability (0.5) of residing at temperatures  $> 0.5^{\circ}\text{C}$ . Comparison of the EDFs showed that 3 of the 4 species spent time in a unique range of water temperatures indicating that *N. coriiceps*, *C. wilsoni* and *L. squamifrons* did not move about randomly in the thermal gradient and thus lends itself to further statistical treatment of the data to determine whether the three species had a unique thermal preference.

The pairwise, two sample Kolmogorov-Smirnov test between each species' EDF which compares the largest distance between the two pooled temperature time series distributions, indicated that each species' temperature/time series data was significantly different (Table 2) (KS-test,  $p$  - value  $< 0.05$ ). The largest distance in the cumulative distribution functions (CDF) was between *N. coriiceps* and the other 3 species while the smallest distances were between comparisons of *T. hansonii*, *C. wilsoni* and *L. squamifrons* (Table 2).

### *Initial to final temperature range analysis*

Comparison of the temperature time series data of ( $T_0$ ) the 500 second and ( $T_{\text{end}}$ ) the last 500 seconds in the gradient indicated their temperature ranges were insignificantly different between the beginning and ending periods for *T. hansonii*, *C. wilsoni* and *N. coriiceps* (Table 3). It

appears that *L. squamifrons*, *C. wilsoni* and *N. coriiceps* had settled into a more limited temperature range than *T. hansonii* which exhibited a much broader temperature range (Fig.5).

#### *Final temperature range comparison between species*

Comparing the  $T_{\text{end}}$  data distribution (Fig. 6) between species using the Kruskal-Wallis two-side non-parametric test, showed that the medians from each species were significantly different ( $\chi^2 = 6181.3$ ,  $p$  - value < 0.05). Post hoc analysis of species  $T_{\text{end}}$  ranges indicated significant differences.

#### *Final temperature range differences between species*

To identify which species differed in their  $T_{\text{end}}$  temperature range and how different was that difference, the Dunn's Kruskal-Wallis Multiple Comparisons post hoc test was performed, demonstrating that the median of the  $T_{\text{end}}$  temperature range was significantly different for each species (Table 4 & Fig. 6) rejecting the null hypothesis that in each comparison the probability of finding a random value in the first group is larger than a random value in the second group is equal to 0.5 (Dinno, 2015). These results suggest that each species has a different preferred temperature range. Thus, with each median being significantly different, the  $T_{\text{end}}$  temperature range revealed that *L. squamifrons* had the highest median temperature at 0.62°C with a relatively small Interquartile Range (IQR), IQR= 0.61°C. *T. hansonii* had the next highest (median = 0.56°C, IQR =1.76°C), followed by *C. wilsoni* (median =0.23°C, IQR = 1.22°C) and *N. coriiceps* with the lowest median and the smallest IQR (median = -0.77°C, IQR= 0.45°C) (Table 5).

#### *Catch site water column thermal profiles*

From XBT casts at *L. squamifrons* catch sites during the 2014 austral winter cruise, water column thermal profiles, at Bismark Strait and Gerlache Strait, confirmed the existence of water temperatures at 0°C or above at depths greater than 150 meters; however, in Dallman Bay the water temperature deviates from this pattern temperature pass ~150m (Fig. 7).

#### *Year round catch site water column temperature profiles*

Water temperature profile data from the World Ocean Database, grouped by month and averaged per standard depth increment within a radius of 1.0 decimal degrees (111.32 km) of catch site showed that water temperatures around Dallman Bay, Gerlache Strait, Hugo Island Basin and Bismark Strait are below 0°C between 0 to 100 meters and 0 to 200 meters between April and December but, are non-freezing year round below 200m with pockets of colder water <0°C

interspersed at lower depths >600 m (Fig. 8 - Top). Year-round water temperature variation per standard depth at catch sites shows higher variation in monthly temperatures at Dallman Bay and Gerlache strait below 300 to 400 m compared to Hugo Island Basin and Bismarck Strait which have less variable water temperatures throughout the water column for each month (Fig. 8 - Bottom).

## DISCUSSION

Hysteresis determinations (Fig. 2) confirmed early reports that *L. squamifrons*, the Antarctic grey rockcod, lacks significant antifreeze activity as indicated by a hysteresis value of only 0.017°C which is near the lower detection limit for determining the difference between the melting point and freezing point with the Nanoliter Osmometer. The serum samples that had the greatest hysteresis also showed slight faceting of the seed crystal resulting in an irregular hexagon. For those samples with minimal hysteresis, generally no faceting was observed and the seed disc simply increased in diameter.

The blood osmolality of *L. squamifrons* specimens from the WAP was  $429 \pm 19$  mOsm/Kg, equivalent to a freezing point depression of  $0.8 \pm 0.0036^\circ\text{C}$  (Fig. 2), which is much lower than what has been reported for high latitude Antarctic notothenioids (550-625 mOsm/Kg) (DeVries and Wohlschlag, 1969; Eastman, 1993) and also 100 mOsm/Kg lower than most antifreeze fortified WAP notothenioid species (Bilyk and DeVries, 2009), but higher than average temperate marine fish osmolality (320-380 mOsm/Kg) (Olson, 1985).

The *L. squamifrons*' combined freezing point depression due to both non-colligative and colligative freezing points is only 0.817°C, one degree higher than both the freezing point of seawater (-1.9°C) and the typical freezing point of most of the Antarctic notothenioid blood sera (Bilyk and DeVries, 2009; DeVries and Wohlschlag, 1969). This result corroborates previously reported values for *L. squamifrons* blood serum freezing points (-0.8°C) (DeVries & Lin 1977; Miya et al. 2016) indicating the exclusion of an elevated interstitial fluid osmolality as a mechanism for freeze avoidance. Furthermore, occasional observations indicated that specimens could freeze in -1.5°C running seawater that was ice-free. The source of nucleation is unclear but it may be that other freeze avoidant species in the tank carried embedded ice crystals in their skin mucus and contact with *L. squamifrons*' nucleated their supercooled body fluids.

To test the hypothesis that *L. squamifrons* uses a behavioral freezing avoidance strategy, specimens along with the AFGP-fortified species (*T. hansonii*, *C. wilsoni* and *N. coriiceps*) were placed in a thermal gradient tank and when comparing the temperature ranges in the last 500 seconds of the 3 hour exposure compared with the first 500 seconds, post acclimation, in the gradient the fish did not exhibit significantly different temperature ranges (Fig. 5 & Table 3). However, each species had a unique temperature range at the end of their time in the thermal gradient (Table 4 & Fig. 6) and *L. squamifrons* had the highest median temperature (0.62°C),

compared to *T. hansonii* (0.56°C), *C. wilsoni* (0.23°C) and *N. coriiceps* (-0.77°C) (Table 5 and Fig. 6). Furthermore *L. squamifrons* had the second smallest IQR, of 0.66°C, suggesting that it not only preferred warmer temperatures (>0°C) but it is also avoided temperature below 0°C compared to *C. wilsoni* and *T. hansonii* which had slightly colder medians but much broader IQRs (1.76°C and 1.22°C, respectively) and *N. coriiceps* with a subzero median (-0.77°C) and the smallest IQR of 0.45°C (Table 5 and Fig. 6).

Thus, with the assumption that a fish will tend to reside in their preferred temperature range when placed in a thermal gradient and that the preferred temperature is one which the organism has evolved to thrive in, the data provides evidence that supports the hypothesis that *L. squamifrons* utilizes a behavioral freeze avoidance strategy. This result is consistent with the observations that this species has always been captured at depths where the benthic water temperature was above 0°C (Fig. 7), and with the presence of non-freezing year-round temperatures at known catch sites below a depth of 200 m (Fig. 8). Together, these data provide support for the preliminary assessment that *L. squamifrons* freeze avoidance strategy, first proposed in 1977, but until now has not been demonstrated.

#### *Thermal preferences of AFGP-fortified species*

Although the thermal preference data on *L. squamifrons* suggests that it relies on behaviorally avoiding freezing by exclusively residing in water at temperatures above its freezing point, the thermal preference data for the AFGP-fortified fish species, *T. hansonii* and *C. wilsoni*, exhibited medians above zero degrees Celsius and broad temperature distributions as indicated by their relatively large IQRs at  $T_{\text{end}}$  (Table 5 and Fig.6). Interestingly, *N. coriiceps* exhibited the narrowest thermal preference range as indicated by its small IQR and the lowest thermal preference (-0.6°C). These findings are consistent with previously reported low critical thermal maxims, a measure of the upper thermal tolerance which a fish loses its ability right itself (Bilyk and DeVries, 2011; Bilyk et al., 2012), which demonstrates that nototheiods have the most limited thermal ranges of any fish and with the our thermal data we propose that nototheiods potentially have the most limited preferred thermal ranges as well.

#### *Advantages of a Behavioral Freeze Avoidance Strategy*

Prior to this study, there were only two reported freeze avoidance strategies found among Antarctic fishes; undercooling (Cziko et al., 2006; Wöhrmann et al., 1997) and AFGP mediated freeze avoidance (DeVries, 1988), but our data supports the existence of a third, behavior based



freeze avoidance strategy. Compared to AFGP mediated freeze avoidance, behavioral freeze avoidance may be an effective compromise between the metabolic demand of maintaining high AFGP concentrations (DeVries, 1988) and the risk of freezing in the undercooled state. Since *L. squamifrons* can survive as an intermediate between those two freeze avoidance strategies, then it may have a reduced energy expenditure compared to AFGP-fortified fish species and this energy saving advantage and may have been why *L. squamifrons* initially diverged from the AFGP based freeze avoidance strategy. However, thus far there have been no experimental studies on the energy expended synthesizing and maintaining high levels of AFGPs in the circulation.

#### *Loss of the AFGP Phenotype*

Our data on *L. squamifrons* non-freezing thermal preference and lack of blood borne AFGPs provides an initial insight into how *L. squamifrons* may have evolved to survive without AFGPs. To arrest ice crystal growth, AFGPs bind to ice crystal surfaces and alter the growth morphology of ice crystals (DeVries 1986), resulting in ice crystals with a hexagonal bipyramidal shape and clearly expressed faces referred to as faceting (Jia et al., 1996). Miya et al. 2016 found that some hexagonal faceting does occur in this species when seed ice crystals are held at temperatures slightly below their equilibrium freezing point however, we found that generally the seed crystals grew as discs without faceting (Fig. 3). Analogous crystal morphologies were reported in AFP ice binding site mutagenic studies which showed that AFPs with amino acid modifications in the ice binding site had significant reductions in faceting (Baardsnes et al., 1999; Jia et al., 1996; Nada and Furukawa, 2012). In addition, these studies also found a correlation between AFP amino acid mutations and decreased TH. The lack of significant TH and absence of faceting suggests that *L. squamifrons*' AFGP genes may have accumulated mutations with time resulting in gene products that lack activity, or the genes are present but not expressed; both explanations could account for the insignificant antifreeze activity.

Evidence for reduction in numbers of AFGP genes or mutated AFGP genes *in vivo* has been demonstrated in two nototheniids which migrated from the freezing Southern Ocean to warmer northern waters several million years ago. Cheng *et al.* (2003) showed that *Notothenia angustata* and *Notothenia microlepidota*, which are endemic to the coastal waters of Southern New Zealand, express small amounts of imperfect AFGP molecules with amino acid substitutions disrupting their primary sequence pattern of Ala/Pro-Ala-Thr. Furthermore, Cheng *et al.* (2003) also reported negligible serum TH values for *N. angustata* comparable to those of *L. squamifrons*

and suggested that notothenioids which migrated to milder climates (where freezing is not a selective pressure) over evolutionary time lose their AFGP genotype. Since *L. squamifrons* has also been able to avoid the selective pressure of freezing through its behavioral freeze avoidance strategy then like, *Notothenia angustata* and *Notothenia microlepidota* it may also have accumulated mutations which resulted in a non-functional AFGP molecule.

The hypothesis that *L. squamifrons* lost its AFGP genes is further supported by previously reported phylogenetic studies by Miya *et al.* (2016) and Lautrédou *et al.* (2012). These studies demonstrate that *L. squamifrons* is paraphyletic to the sub-Antarctic Patagonotothens which are a clade of teleost fish that also lack antifreeze activity and AFGP genes as indicated by the absence of bands in genomic Southern Blots when probed with a labeled antifreeze gene fragment (Miya *et al.*, 2016). However, *L. squamifrons* sister species *Lepidonotothen nudifrons* and *Lepidonotothen larseni* are AFGP-fortified along with other paraphyletic Notothenioid species, which makes it unlikely that both *L. nudifrons* and *L. larseni* independently evolved AFGPs. Together, this suggest that *L. squamifrons*, like the Patagonotothens, experienced a loss of its AFGP genes instead of the alterative hypothesis that it never had them in them first place. Determining the suite of mutations which resulted in the loss of *L. squamifrons* AFGP phenotype would be the next step in understanding how this species came to lose such a seemingly critical trait.

Thus, if mutations lead to the loss of the AFGP genotype then ancestral *L. squamifrons* species may have been able to survive by finding warm layers of water ( $>0^{\circ}\text{C}$ ). As our year round water column profiles and constructed coastal bathythermal maps indicate, the presence of warm water would allow this species to avoid the selective pressure of freezing (Fig 8 and Clarke *et al.* 2009). However, if such mutations may not have significantly increased the risk of freezing due to the presence and prevalence of non-freezing water temperatures why is it that other notothenioids, which also inhabit the same benthic habitat, did not lose their AFGP phenotype?

## CONCLUSION

Our data on *L. squamifrons*' low blood serum osmolality and relatively unmeasurable amounts of TH demonstrates that this Antarctic notothenioid species clearly lacks significant antifreeze activity and with its low blood osmolality precludes elevated blood osmolality as a potential freeze avoidance strategy. Furthermore, our data on *L. squamifrons*' exclusively nonfreezing thermal preference provides evidence, for the first time, that an Antarctic fish is utilizing behavioral freeze avoidance as its primary freeze avoidance strategy. The validity of this strategy is supported by the presence and prevalence of year-round non-freezing water temperatures around the WAP's coastal waters deeper than 200m thereby dispensing undercooling as an alternative freeze avoidance strategy. Thus, *L. squamifrons* behavioral freeze avoidance strategy stands as an exception to the conventional AFGP based freeze avoidance strategies found among Antarctic notothenioids and broadens our understanding of viable strategies that are conducive to survival in the extremely cold marine environments.

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## TABLES AND FIGURES

**Table 1** - Approximate fish catch locations and depths around the WAP during the 2014 season.

Species	n	Catch Location	Latitude	Longitude	Depth (m)
<i>Notothenia coriiceps</i>	6	Palmer Station	-64 44.975	-64 3.126	1-3
<i>Chaenodraco wilsoni</i>	6	Gerlache Strait	-64 43.376	-63 01.005	300-400
<i>Trematomus hansonii</i>	5	Gerlache Strait	-64 43.376	-63 01.005	300-400
<i>Lepidonotothen squamifrons</i>	6	Gerlache Strait	-64 43.376	-63 01.005	150-400
		Hugo Island	-64 41.572	-65 31.952	650-700
		Bismark Strait	-64 52.258	-63 38.02	170-200
		Dallman Bay	-63 54.999	-62 46.372	175-180

**Table 2** - The Pairwise Two-sample Kolmogorov-Smirnov test statistics D and p-values of EDF between species.

Pairwise Species Comparison	<i>N. coriiceps</i>	<i>T. hansonii</i>	<i>C. wilsoni</i>
<i>L. squamifrons</i>	D = 0.87205 p-value < 0.05	D = 0.32544 p-value < 0.05	D = 0.27185 p-value < 0.05
<i>C. wilsoni</i>	D = 0.67049 p-value < 0.05	D = 0.32664 p-value < 0.05	-
<i>T. hansonii</i>	D = 0.62217 p-value < 0.05	-	-

**Table 3** - Wilcoxon rank sum test results (two-sided) for T<sub>0</sub> and T<sub>end</sub> temperature range mediums.

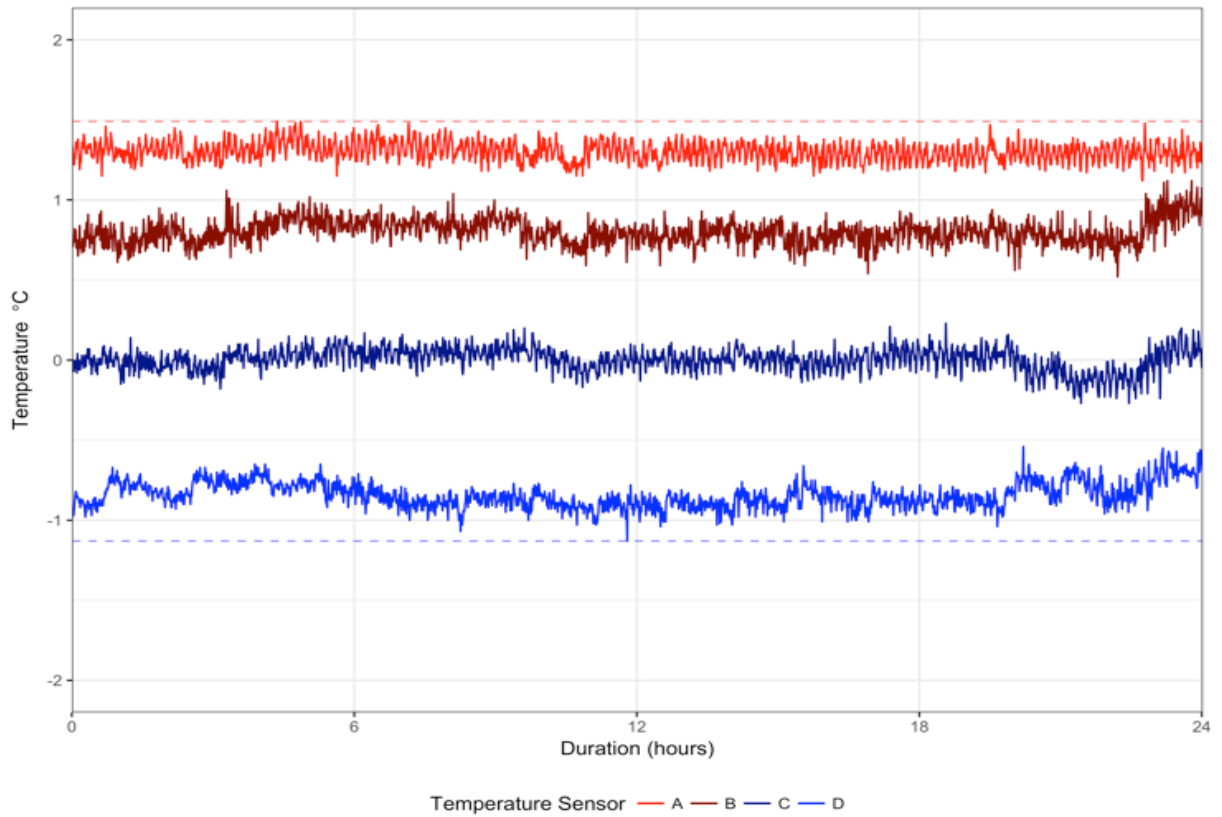
Wilcoxon rank sum test	T <sub>0</sub> vs T <sub>end</sub>
<i>L. squamifrons</i>	p-value = 0.4222
<i>C. wilsoni</i>	p-value = 0.1525
<i>T. hansonii</i>	p-value = 0.5159
<i>N. coriiceps</i>	p-value = 0.1967

**Table 4** - Dunn's Kruskal-Wallis two-sided multiple comparison with p-values adjustments made with the Bonferroni method

Dunn's Multiple Comparison	<i>N. coriiceps</i>	<i>T. hansonii</i>	<i>C. wilsoni</i>
<i>L. squamifrons</i>	Z= 69.24 p – value adj. < 0.05	Z = 5.07 p – value adj. < 0.05	Z= -12.59 p – value adj. < 0.05
<i>C. wilsoni</i>	Z = 56.65 p – value adj. < 0.05	Z = -7.51 p – value adj. < 0.05	-
<i>T. hansonii</i>	Z = -64.16 p – value adj. < 0.05	-	-

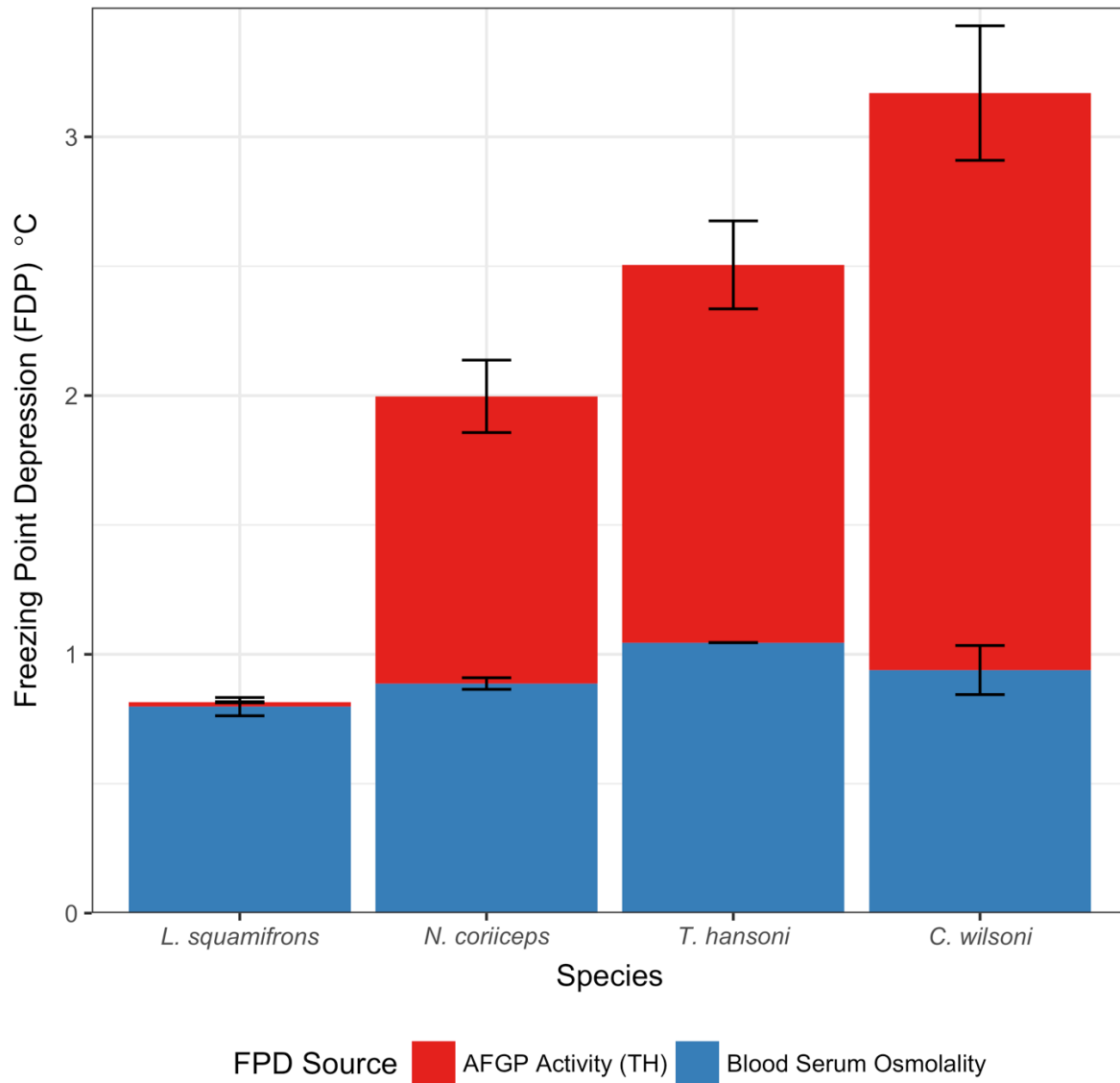
**Table 5** - Summary statistics for each species' T<sub>end</sub> and the Interquartile Range (IQR), in degrees Celsius.

Species	Minimum	1 <sup>st</sup> Quartile	<b>Median</b>	3 <sup>rd</sup> Quartile	Maximum	<b>IQR</b>
<i>L. squamifrons</i>	0.01	0.23	0.61	0.89	1.33	<b>0.66</b>
<i>T. hansonii</i>	-0.77	-0.21	0.56	1.54	1.54	<b>1.76</b>
<i>C. wilsoni</i>	-0.43	-0.32	0.23	0.89	1.22	<b>1.21</b>
<i>N. coriiceps</i>	-1.11	-1.00	-0.77	-0.54	-0.43	<b>0.45</b>

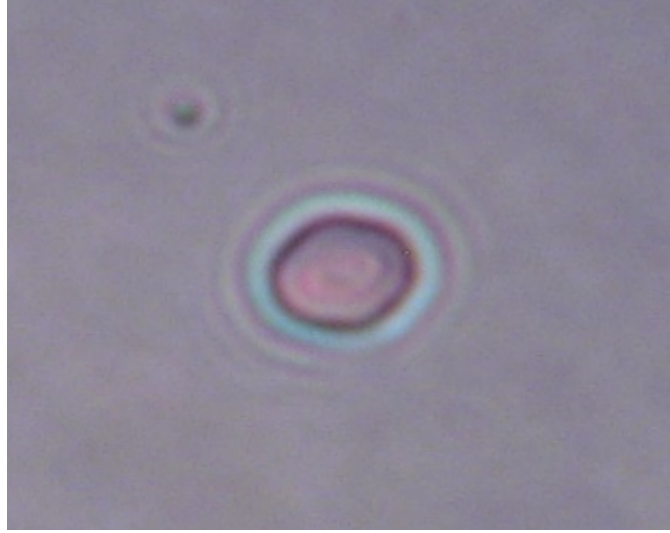


**Figure 1** - Recorded temperature in the thermal gradient tank over a period of 24hrs, with four temperature sensors located 107cm apart starting from the warm to the cold end of the tank. The dashed red and blue lines indicate the maximum upper and lower minimum temperature limits of the thermal gradient tank, which will be used in subsequent graphs to demark the testing limits of the thermal gradient tank.

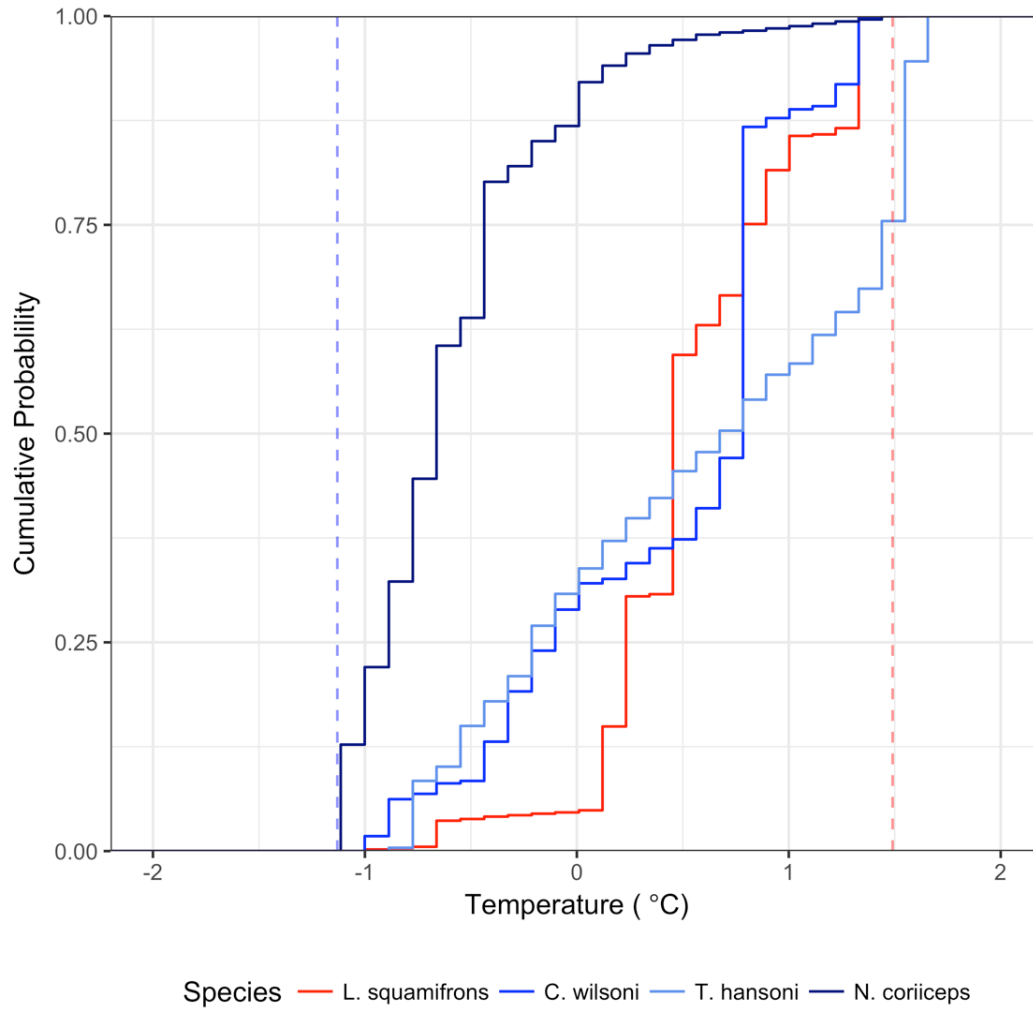




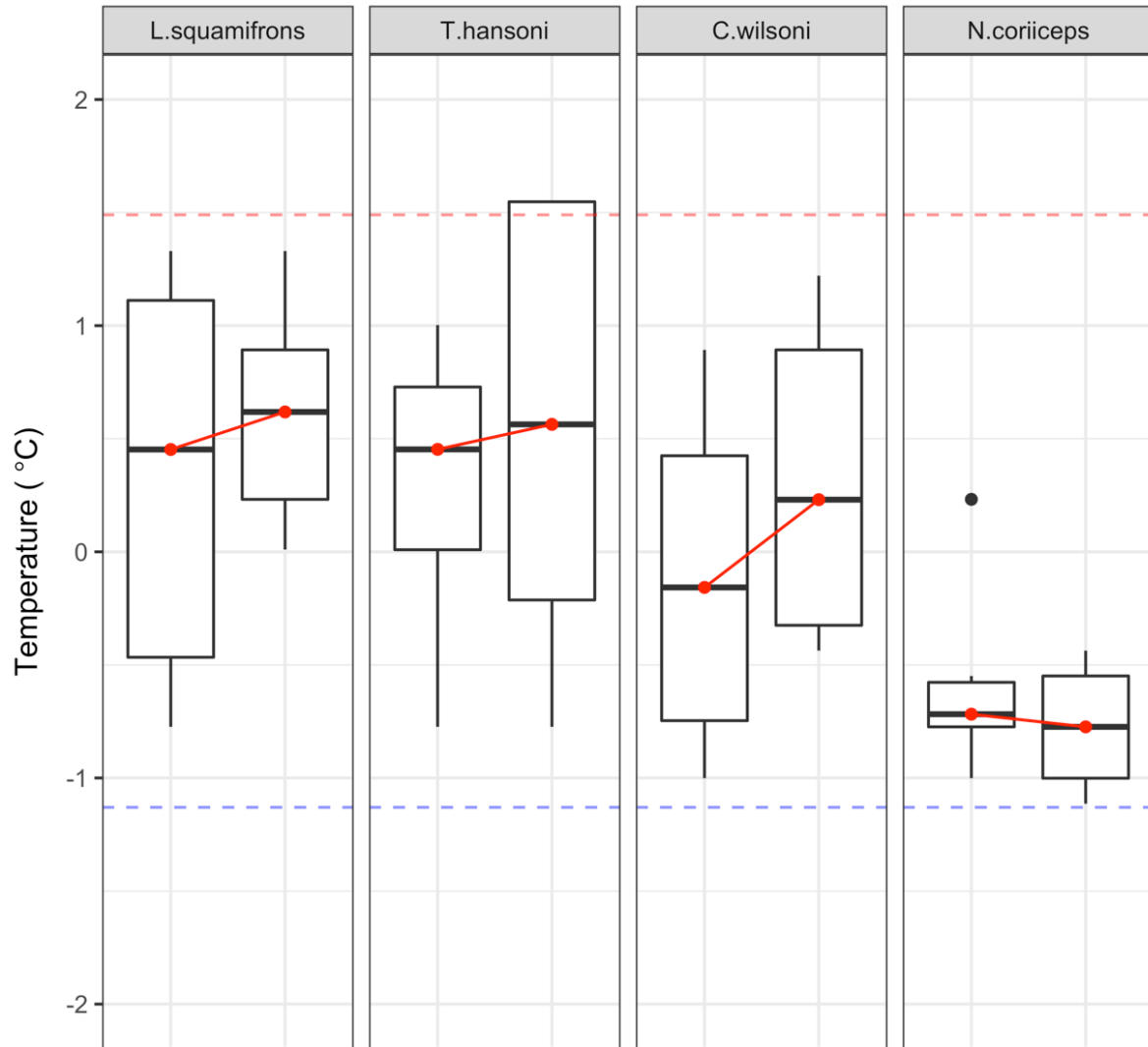
**Figure 2** - Blood serum osmolality and thermal hysteresis (mean with SE) of *C. wilsoni* (Bilyk and DeVries, 2009), *T. hansonii* (Jin, 2003; O’Grady et al., 1982), *N. coriiceps* (Jin and DeVries, 2006) and *L. squamifrons* (n=18).



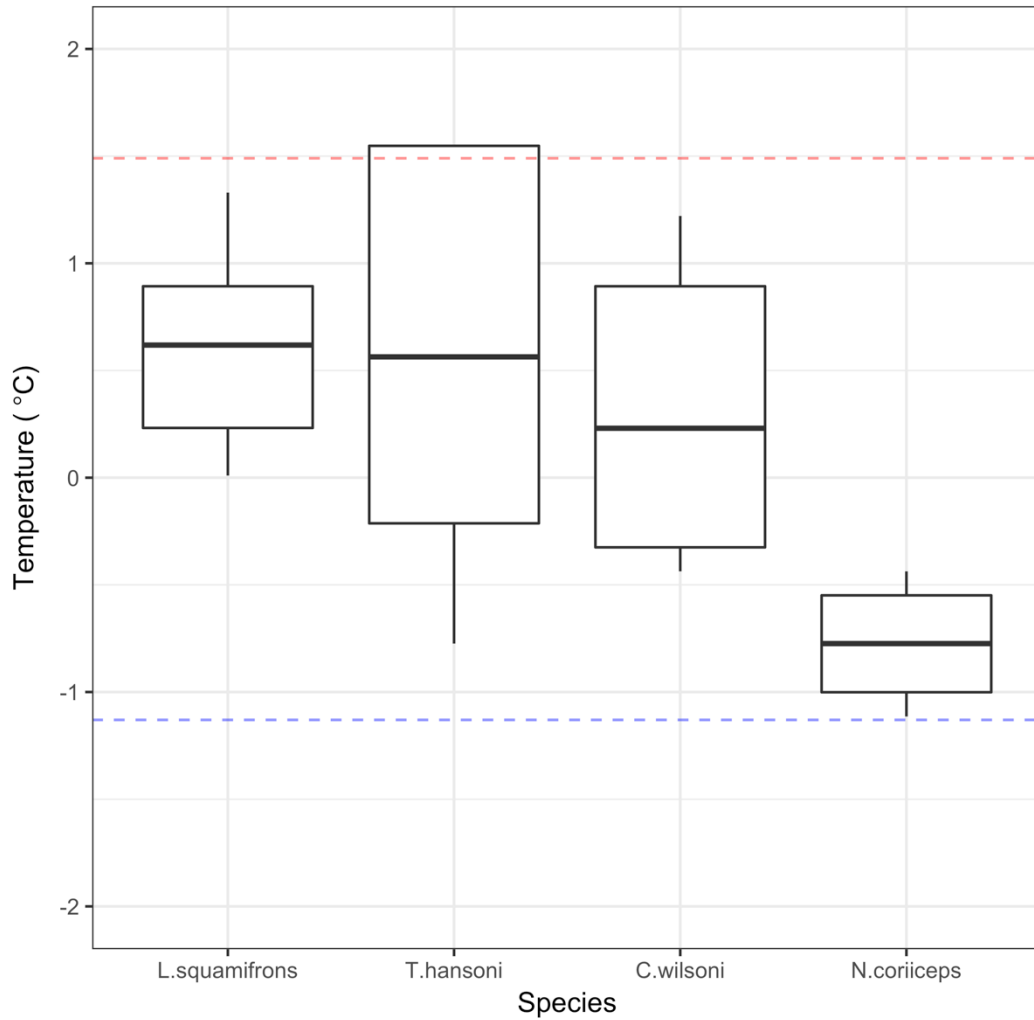
**Figure 3** - A representative photo of *L. squamifrons*' blood serum ice crystal ~10µm diameter.



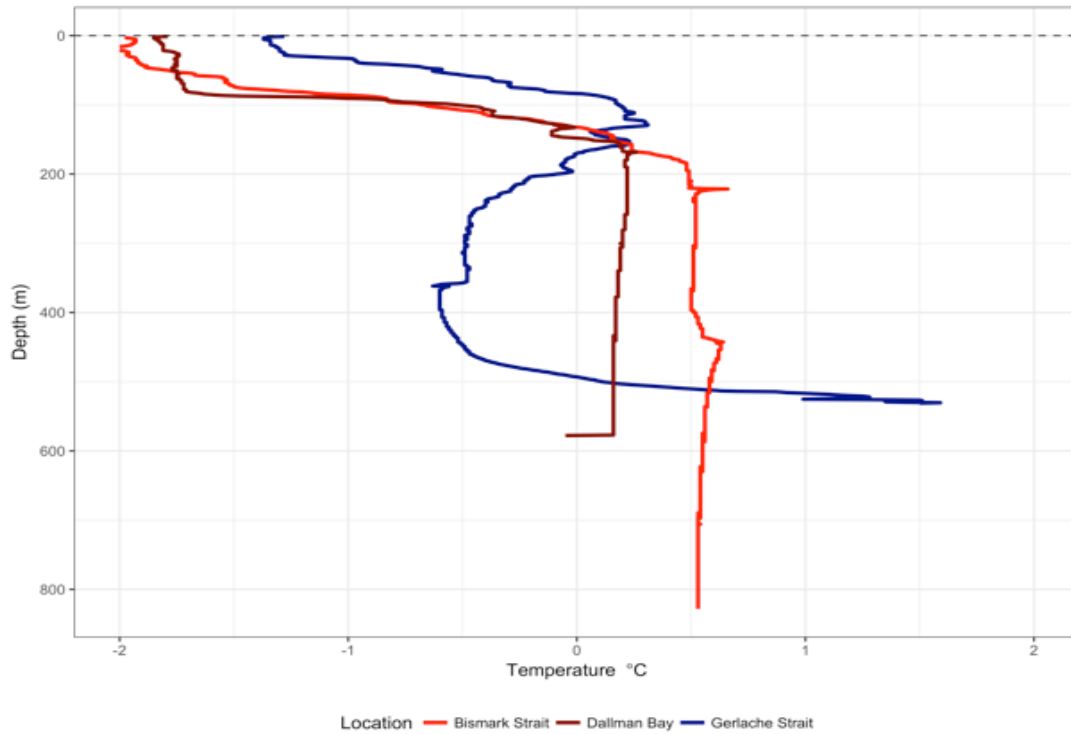
**Figure 4** - EDF plot of each species pooled time series data. The dashed red and blue lines indicate the maximum upper and lower minimum temperature limits of the thermal gradient tank.



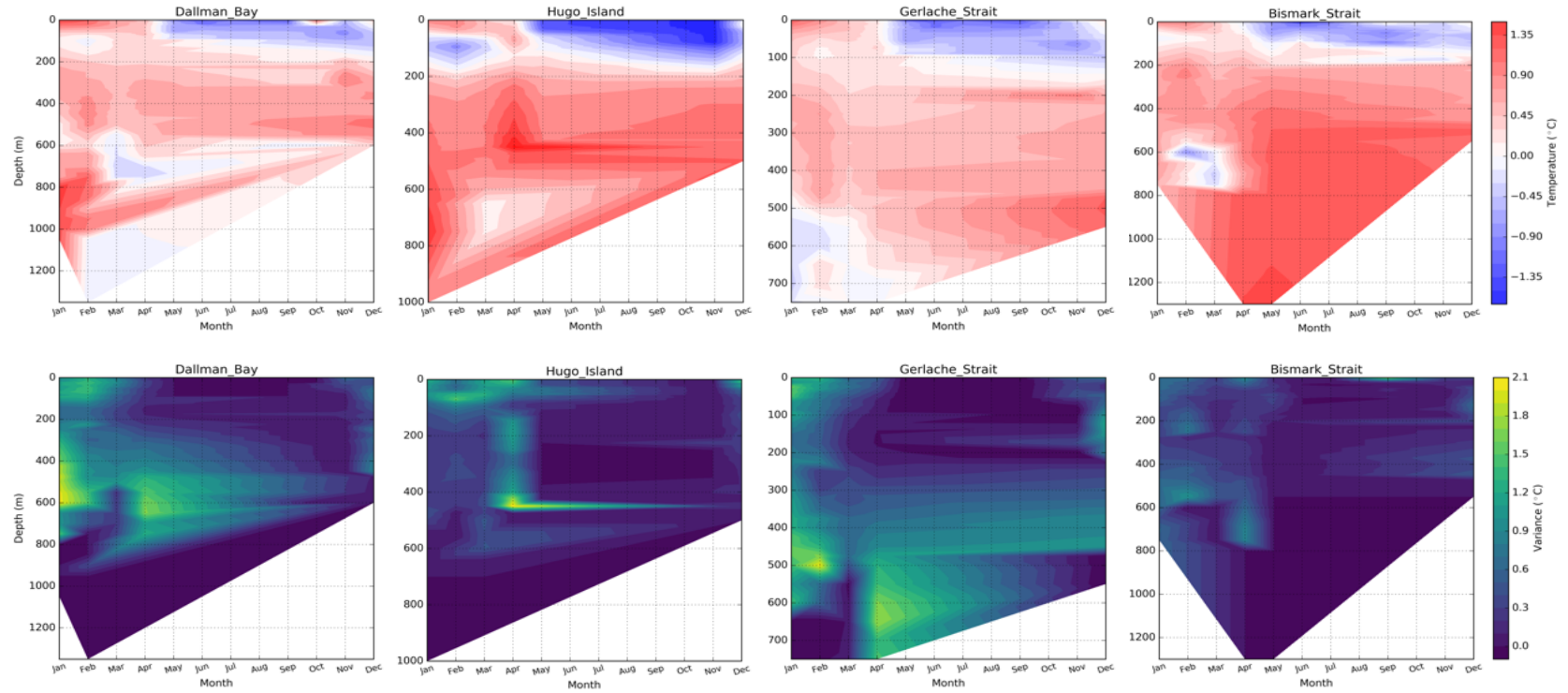
**Figure 5** -  $T_0$  and  $T_{end}$  distributions for each species. The dashed red and blue lines indicate the maximum upper and lower minimum temperature limits of the thermal gradient tank.



**Figure 6** - Median and box plots for  $T_{end}$  distributions used to show the thermal preference for each species. The dashed red and blue lines indicate the maximum upper and lower minimum temperature limits of the thermal gradient tank.



**Figure 7** - Onsite XBT temperature profile of the water column at locations where *L. squamifrons* was caught. Location coordinates for each measurement: Bismark Strait (-64 52.45801, -63 39.5166) Dallman Bay (-63 55.85107 -62 48.50049), Gerlache Strait (-64 43.35596, -63 1.41016).



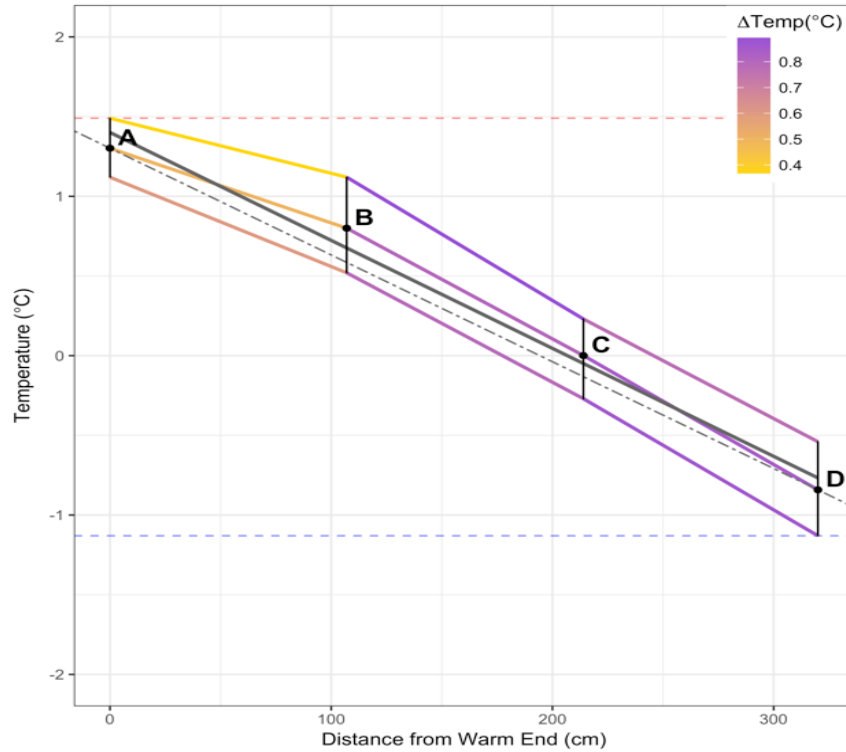
**Figure 8** - (Top row) Year-round water column thermal profile (C) generated from historical data within 1 decimal degree radius of catch site locations. (Bottom row) Variance of year-round water column thermal profile data for each site.

## APPENDIX A

**Appendix A: supplemental Table 1-** Blood serum osmolality and thermal hysteresis (mean with SE) (*Bilyk and DeVries, 2009; Grady and Devries, 1982; Jin, 2003; Jin and DeVries, 2006*)

Species	Blood Serum Osmolality (mOsm/Kg)	Thermal Hysteresis (°C)
<i>N. coriiceps</i>	477 ± 12	-1.11 ± 0.14
<i>C. wilsoni</i>	505 ± 51	-2.23 ± 0.26
<i>T. hansonii</i>	562	-1.46 ± 0.17
<i>L. squamifrons</i>	429 ± 19	-0.017 ± 0.0021





**Appendix A: Supplemental Figure 1** - Mean, minimum and maximum temperatures of the thermal gradient over a 24-hour period taken at 107cm intervals along the tank, with differences in temperature between sensors colored according to the legend. Solid grey line is the linear fit to the data with the dashed grey line representing a one to one relationship between the end points of the gradient. Dashed blue and red lines represent overall minimum and maximum temperatures. Change in temperature between thermocouples A and B ( $\Delta AB = 0.51^{\circ}\text{C}$ ) is smaller than the change in temperatures between B & C ( $\Delta BC = 0.79^{\circ}\text{C}$ ), C & D ( $\Delta CD = 0.84^{\circ}\text{C}$ ). Together this shows, between sensors B, C and D the temperature decreased at a similar rate while between sensors A & B the temperature decreased slower which means the temperature gradient was not uniformly distributed between the warm and cold ends of the tank.